Chapter 5
Physiology and Evolution of Wing Pattern Plasticity in *Bicyclus* Butterflies: A Critical Review of the Literature

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Abstract Phenotypic plasticity refers to the ability of a genotype to develop into different phenotypes in response to environmental cues. In many instances, this ability is an evolved adaptation to enable organisms to adapt to predictable but variable environments in time or space (West-Eberhard MJ, Developmental plasticity and evolution. Oxford Unversity Press, New York, p 794, 2003; Stearns SC, BioScience 39(7):436–445, 1989; Bradshaw AD, Evolutionary significance of phenotypic plasticity in plants. In: Caspari EW (ed) Adv Genet 13. Academic, New York, pp 115–155, 1956; de Jong G, New Phytol 166(1):101–117, 2005; Moran NA, Am Nat 139(5):971–989, 1992). While much research has focused on the ecological and adaptive significance of the alternative phenotypes produced under different environments, relatively little is still known about the proximate physiological and molecular mechanism translating environmental variation to phenotypic variation and how these mechanisms may have evolved (Beldade P, Mateus ARA, Keller RA, Mol Ecol 20(7):1347–1363, 2011).

Here I provide a review of the literature that has explored how environmental variation, in particular seasonal variation, impacts eyespot size in African satyrid butterflies of the genus *Bicyclus*. Plasticity in eyespot size is undeniably the most conspicuous effect of seasonal variation on the appearance of *Bicyclus* species, and perhaps because of this, its ecological and physiological bases have been under investigation since 1984 (Brakefield PM, Reitsma N, Ecol Entomol 16:291–303, 1991; Brakefield PM, Larsen TB, Biol J Linn Soc 22:1–12, 1984). Much subsequent research on members of this genus, and in particular on the model species *Bicyclus anynana*, uncovered, however, many other morphological, behavioral, physiological, and life history traits that are equally impacted by seasons and, in particular, by rearing temperature (Bear A, Monteiro A, Plos One 8(5), 2013; Dion E, Monteiro A, Yew JY, Scientific Reports 6:39002, 2016; Fischer K, Brakefield PM, Zwaan BJ, Ecology 84(12):3138–3147, 2003a; de Jong MA, Kesbeke F,

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Brakefield PM, Zwaan BJ, Climate Res 43(1–2):91–102, 2010; Mateus ARA, Marques-Pita M, Oostra V, Lafuente E, Brakefield PM, Zwaan BJ, et al. Bmc Biology 12, 2014; Windig JJ, Brakefield PM, Reitsma N, Wilson JGM, Ecol Entomol 19:285–298, 1994; Fischer K, Eenhoorn E, Bot AN, Brakefield PM, Zwaan BJ, Proc R Soc B 270(1528):2051–2056, 2003b; Everett A, Tong XL, Briscoe AD, Monteiro A, BMC Evol Biol 12:232, 2012; Prudic KL, Jeon C, Cao H, Monteiro A, Science 331(6013):73–75, 2011; Westerman E, Monteiro A, Plos One 11(2), 2016; Macias-Munoz A, Smith G, Monteiro A, Briscoe AD, Mol Biol Evol 33(1):79–92, 2016). This review, however, focuses solely on eyespots, the original trait that initiated explorations of phenotypic plasticity in this butterfly genus.

5.1   Introduction

Insects have relatively short lives, and this promotes the evolution of seasonal forms or polyphenisms. A short life means that insects can live all their lives within a particular season, in regions of the world that have seasons. This also means that cohorts that emerge in different seasons (spring or summer or wet or dry seasons) will encounter very different biotic and abiotic environments. These environments often exert different selection pressures on the appearance of these insects in order to enhance their survival and reproduction in the respective season. The evolution of adaptive phenotypic plasticity is then a natural response to these predictable, recurrent, but alternate environments that different cohorts of insects experience at different times of the year. This type of plasticity is called a seasonal polyphenism and is especially notable in the highly conspicuous wing patterns of butterflies that inhabit seasonal environments (Brakefield and Larsen 1984; Nijhout 1999, 2003).

One type of wing pattern in butterflies that is especially sensitive to seasonality is the eyespot pattern. Eyespots found in the exposed surfaces of the wings (most of the ventral wing surfaces) are often large in the wet season (WS) and small in the dry season (DS) in the African tropics (Brakefield and Larsen 1984), as well as in many other regions of the world (Fig. 5.1). The ecological significance of this plasticity has been explored with a variety of experiments in the field (Brakefield and Frankino 2009; Ho et al. 2016) and in the lab (Lyytinen et al. 2003, 2004; Prudic et al. 2015; Olofsson et al. 2013; Vlieger and Brakefield 2007). The consensus, so far, is that small cryptic eyespots are an adaptation of the butterfly to avoid being detected by vertebrate predators, who predominate in the DS (Lyytinen et al. 2003), whereas the more conspicuous eyespots are an adaptation to deflect the attacks of invertebrate predators, such as mantids, who predominate in the WS (Prudic et al. 2015).
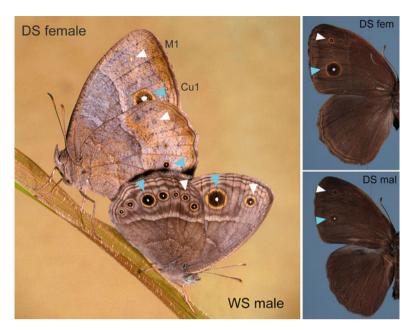
Fig. 5.1 Patterns of plasticity in *Bicyclus anynana* butterflies. Main image depicts a DS female (*left*) mating with a WS male. Eyespots described in this review are named M1 (*white arrow*) and Cu1 (*blue arrow*). The ventral wing surfaces are often exposed to predators with the exception of the Cu1 forewing eyespot, which is often hidden by the hindwing. The *right panels* depict the hidden (*dorsal*) surfaces of a DS female (*top*) and a DS male displaying sexual dimorphism in their Cu1 eyespots

Butterfly eyespots that are found in hidden (mostly dorsal) surfaces have different patterns of plasticity altogether because these eyespots serve different functions in each of the seasons. These eyespots are used in sexual signaling by both sexes (Prudic et al. 2011; Robertson and Monteiro 2005; Costanzo and Monteiro 2007) (Fig. 5.1). Males use these eyespots to signal to females in the WS, and females use the same eyespots to signal to males in the DS. This leads to patterns of size plasticity that are congruent with those from ventral surface eyespots for males (large in WS males and small in DS males) but not for females. DS females, in particular, have abnormally large dorsal eyespots, which they use for sexual signaling to males in this season (Fig. 5.1), which are at odds with the small size of their ventral exposed counterparts. Females, thus, don't display size plasticity in these eyespots – they are large in both seasons. The patterns of sexual selection operating on dorsal eyespots lead to sexual size dimorphism in dorsal Cu1 eyespots in the DS (Fig. 5.1), as well as a male-specific pattern of plasticity for these eyespots (Bhardwaj et al. 2017).

The review that follows looks critically at the literature that has investigated the environmental, physiological, and molecular mechanisms that regulate eyespot size plasticity in both dorsal and ventral eyespots. In addition, the evolution of phenotypic plasticity in eyespot size is also reviewed.

5.2 Physiological Mechanisms of Eyespot Plasticity

Bicyclus anynana is found from Ethiopia to South Africa (Condamin 1973) and has evolved along a range of climates, but the original lab population of *Bicyclus anynana* stems from Malawi, a country with strong seasonality. The arrival of the dry season in Malawi is primarily cued by decreasing temperatures, whereas the arrival of the wet season is cued by increasing temperatures (Brakefield and Reitsma 1991). Lab-rearing experiments, where photoperiod and thermoperiods were varied, confirmed that average temperature and fluctuations in night- and daytime temperature were the most important determinants of eyespot size plasticity in this species (Brakefield and Mazzotta 1995). Food plant quality, however, also affected eyespot size plasticity (Kooi 1995).

Once environmental cues with significant effects on the induction of plasticity were identified, the next investigations probed how and when these cues interacted with the gene regulatory networks that differentiate the eyespot patterns to modify their output in a plastic manner. In particular, these investigations focused on the mechanisms whereby average daily temperature induced the wet and the dry seasonal forms in *B. anynana*.

The first consideration was whether temperature only exerted its effects on wing pattern development during specific developmental windows or critical temperature-sensitive stages. Early work in this system used temperature-shift experiments to identify the critical period during eyespot development that was sensitive to rearing temperature and able to modify the final size of eyespots (Kooi and Brakefield 1999). These experiments used a variety of shifts differing in length of time that the animals were kept at each of the two alternative temperatures (17 and 27 °C) and times of initiation of the shift. Kooi and Brakefield (1999) concluded that the most important period of sensitivity that led to changes in the size of two of the ventral eyespots (forewing M1 and hindwing Cu1 eyespots) was the final 5th larval instar. Furthermore, while they found that temperatures experienced during the first 24 hrs of pupal development still impacted eyespot size, they concluded that temperatures experienced during this period could not shift a WS wing pattern into a DS pattern and vice versa (Kooi and Brakefield 1999).

More recent work replicated these experiments, with narrower window temperature shifts, and confirmed that the late larval period, in particular, the wandering stage of development, when the larvae stop eating and start looking for a place to pupate, was the most temperature-sensitive stage for the determination of ventral eyespot size plasticity of Cu1 ventral hindwing eyespots (Monteiro et al. 2015). These experiments also highlighted that forewing and hindwing ventral Cu1 eyespots in females responded differently to temperature. Forewing Cu1 eyespots, which are normally hidden by the hindwing when the butterfly is at rest (Fig. 5.1), were much less plastic than Cu1 hindwing eyespots, which are always exposed at rest. In addition, the size of the white center in forewing eyespots was not plastic at all (Monteiro et al. 2015). Subsequent work (Bhardwaj et al. 2017), examining plasticity in dorsal eyespots, similarly concluded that the wandering

stage is the most temperature-sensitive stage for male eyespots (female eyespots are not plastic). In summary, eyespot size is primarily sensitive to temperature during the wandering stages of development, but size of Cu1 serial homologous eyespots on ventral forewings and hindwings does not respond to temperature in the same way.

Most examples of phenotypic plasticity known from insects seem to rely on a hormonal signal to translate variable environments into variable phenotypes (Nijhout 1999, Beldade et al. 2011). This prompted the search for the hormones responsible for the variation in wing pattern across *B. anynana* seasonal forms. Previous work on two different butterflies, the map butterfly *Araschnia levana* and the buckeye *Junonia coenia*, had discovered that differences in the presence and absence of a peak of the molting hormone, 20-hydroxyecdysone (20E), during the early pupal stage explained the different seasonal forms (spring and summer forms) of these butterflies, displaying different wing colors in response to day length (an important environmental cue used for regulating plasticity in these systems) (Koch and Buckmann 1987; Nijhout 1980; Rountree and Nijhout 1995). 20E became, thus, a candidate hormone to be investigated in connection with eyespot size plasticity in *B. anynana*.

Surprisingly, early work surrounding investigations into the physiological basis of eyespot size plasticity decided not to investigate physiological differences between the seasonal forms but instead focus on physiological differences observed between lines reared at the intermediate temperature of 20 °C, whose eyespots had been artificially selected to mimic the dry and wet season forms (Brakefield et al. 1998; Koch et al. 1996). In addition, titers of 20E were measured in individuals of these WS and DS form "genetic mimics" at different stages of development focusing primarily in the early pupal stages, as no differences were observed between these mimics during the wandering stages (Koch et al. 1996). Titers of 20E measured in the early pupal stage showed small differences between the seasonal form genetic mimics, and 20E injections into the dry season form mimic, which had a natural slower increase of 20E during the pupal stage, showed small (albeit significant) increases in eyespot size toward the phenotype of wet season forms (Koch et al. 1996). Later work, however, showed that these 20E titer differences observed between WS and DS form genetic mimics could more readily explain variation in pupal stage duration than eyespot size differences (Oostra et al. 2011).

Recent work finally measured 20E hemolymph titers in late larvae of temperature-induced WS and DS forms and discovered that levels of 20E differed significantly between the seasonal forms during the wandering stage of development (Monteiro et al. 2015). This is important because this stage of larval development is contained within the 5th and final larval stage, previously identified as the temperature-sensitive period for induction of eyespot size plasticity (Kooi and Brakefield 1999; Monteiro et al. 2015). Levels were higher in WS forms relative to DS forms, indicating a positive correlation between 20E and eyespot size.

To test whether these different levels in 20E were causing the variable wing phenotypes, hormone injections and hormone receptor manipulations were both

done. These two types of manipulations, however, are not equivalent, but this has remained unrecognized by many researchers in this field (but see Zera 2007). To test whether the presence of a hormone at a given level is leading to the development of a phenotype, removal of the hormone or its producing cells/organs, or interfering with its specific receptor, are the best type of manipulations to test causation. If this cannot be done, adding hormone to the form with the lower natural levels to mimic the form with the highest levels is also possible. This latter type of manipulation, however, is more challenging to do because levels of the added hormone need to mimic rather than exceed the highest natural levels found in any of the plastic forms. If levels exceed the natural levels, this may lead to abnormal phenotypes that play no role in normal trait development. One way these abnormal phenotypes may emerge is if raising the levels of hormone A beyond some critical level stimulates the production of hormone B, which then impacts the trait of interest directly. In this situation, manipulations of hormone A would lead researchers to conclude incorrectly that it regulates the trait, when in fact it does so only via its effects on hormone B, which was induced due to high abnormal levels of A. Cross talk between hormonal systems is common, and special attention needs to be paid to this (Zera 2007; Orme and Leevers n.d.).

An example of the type of asymmetry in the response that can be observed with the two types of manipulation experiments described above was observed with 20E signal manipulations in the wandering larval stages of *B. anynana*. As mentioned above, WS wanderers have higher levels of 20E relative to DS wanderers. In order to test whether 20E levels at this stage of development were regulating adult eyespot size, injections of cucurbitacin B (CurcB), a EcR receptor antagonist (Dinan et al. 1997), and a control vehicle, were performed in WS wanderers to test whether they led to reduced adult eyespot size (Monteiro et al. 2015). CurcB is a small molecule that binds with high affinity to the ecdysone receptor (EcR), preventing 20E from binding it and preventing downstream signaling from taking place (Dinan et al. 1997). Injecting CurcB into WS forms led to adult butterflies exhibiting small eyespots resembling DS forms (Monteiro et al. 2015). However, Cu1 ventral forewing eyespots, which are less plastic than their Cu1 ventral hindwing counterparts, did not change in size. The asymmetry in the response of the two Cu1 eyespots to CurcB injections can be explained because the EcR receptor is present in Cu1 forewings eyespot centers but is absent in Cu1 hindwing eyespot centers (Monteiro et al. 2015). Absence of the receptor in forewing eyespot centers essentially makes them insensitive to the CurcB manipulation. What is important to note, however, is that these forewing eyespots, despite expressing no EcR, responded to injections of 20E and increased in size, just like their hindwing counterparts that expressed EcR. One possibility is that if 20E levels attained in DS forms via injections were beyond those observed in WS forms, they may have stimulated the production of a second hormone, which also contributed to the regulation of hindwing eyespot size via its own receptor.

To understand how temperature (and hormones) affected eyespot development, Brakefield et al. (Brakefield et al. 1996) looked at an early marker of eyespot development, the transcription factor *Distal-less (Dll)*, in late larvae and in early

pupae. Dll showed comparable expression domains in 5th instar larval wings but had a broader domain of expression in the eyespot centers of WS forms in the early pupal stage. In addition, this gene also had a second domain of expression that corresponded to the much broader black disc of scales in an eyespot, which became visible later, around 12 h after pupation (Brunetti et al. 2001a; Monteiro et al. 2006). The larger group of cells expressing Dll clustered in the eyespot center, however, suggested that some time in between the late larvae and early pupal stages, the eyespot centers were becoming larger in response to temperature. A subsequent study looked at two other markers for eyespot development and found that Notch and Engrailed genes were expressed earlier in the eyespot centers of DS forms relative to their later expression in WS forms, suggesting that these genes could be downregulating eyespot size in DS forms (Oliver et al. 2013). The onset of Dll expression in the eyespot centers of WS and DS forms, however, was approximately the same (Oliver et al. 2013). A more recent study (Bhardwaj et al. 2017) showed that a fourth gene expressed in eyespot centers, the ecdysone receptor (EcR), showed an enlargement in its domain of expression during the second half of the wandering stage in WS forms. Cells in the center of dorsal forewing eyespots underwent cell division concurrently with the rise of 20E titers taking place at that stage of development. Other marker genes, such as Spalt, also increased their domains of expression at the same time, concurrently with local cell divisions. Cells in the dorsal eyespot centers of DS males, however, experiencing the lowest levels of 20E hormone, did not undergo cell division and produced a small eyespot center as well as an associated small eyespot. To test whether levels of 20E were directly responsible for the regulation of dorsal eyespot center size via a localized process of cell division, injections of 20E (into DS males) and CurcB (into WS forms) at 60% of wandering stage development were performed and confirmed an effect of 20E levels on the regulation of eyespot center sizes in WS individuals as well as in DS females, the odd sex with the abnormally large eyespots (Bhardwaj et al. 2017).

The experiments above pin the critical stage of regulation of eyespot center size, and eyespot size for both dorsal and ventral eyespots, to the second half of the wandering stage of development. At this stage, rearing temperature leads to variation in 20E titers, which in turn leads to localized patterns of cell divisions in cells that express the EcR receptor (Bhardwaj et al. 2017). These localized patterns of cell division determine the size of the eyespot centers, which are critical determinants of the size of the complete eyespot pattern (Monteiro and Brakefield 1994), and thus impact final eyespot size.

For many years, however, research into the physiological and genetic basis of eyespot size plasticity focused exclusively on the period of development following pupation, which is not as sensitive to temperature as the previous larval wandering stage (Kooi and Brakefield 1999; Monteiro et al. 2015). This period shows variation in timing of 20E titers in the seasonal form "genetic mimics" as well as in the actual seasonal forms (Mateus et al. 2014; Oostra et al. 2011). In particular, titers of 20E are low during the first 24 h (WS) (and 48 h in the DS) after pupation, which is the developmental window believed to be important for eyespot ring differentiation at

high temperatures (French and Brakefield 1992; Brunetti et al. 2001b). This period of low hormone titers is followed by steadily rising 20E titers, where titers raise earlier in WS than in DS forms, relative to total development time. Furthermore, injections of large quantities of 20E (0.1 ug) into young pupae (0–6 h old) reared at 20 °C led to no changes in eyespot size (Koch et al. 1996). Eyespot size changed slightly only with injection of 20E doses larger than 0.25 ug at this early pupal stage (Koch et al. 1996). Note that injections of merely 0.006 ug of 20E (a dose that is 16 times smaller than 0.1 ug) into wanderers reared at 17 °C were sufficient to produce an almost complete seasonal form reversal in this butterfly species (Monteiro et al. 2015).

More recent experiments, focusing again on the early pupal stage, remeasured 20E titers in vehicle-injected and 20E-injected young pupae (3% of pupal development) reared at two different temperatures (19 and 27 °C) and documented small but significant differences in 20E hormone titers between vehicle-injected seasonal forms right after the injections (at 3.5% of pupal development) (Mateus et al. 2014). WS forms had slightly higher titers of 20E than DS forms. Differences in 20E titers in vehicle-injected seasonal forms, however, were no longer present at 8% of pupal development. While these titer measurements are not exactly "baseline" measurements for natural levels of 20E across these two rearing temperatures, they nevertheless show differences in 20E levels across the two seasonal forms (Mateus et al. 2014). In order to test the significance of these differences, injections of 20E were performed into both DS and WS seasonal forms at this early stage (3%) of pupal development, as well as at a later stage (16% pupal development), before the large raise in 20E titers. Special attention was paid to changes in the area of each of the color rings (white center, black, and gold ring) in a variety of different eyespots on dorsal, ventral, forewing, and hindwing surfaces, which are being determined at this stage of development (Brunetti et al. 2001b). One point of concern in these experiments, however, is that injections used 0.25 ug of 20E, a dose previously shown to produce effects on ventral wing patterns (Koch et al. 1996) but also shown to lead to unnaturally high levels of 20E titers in the hemolymph of pupae of both seasonal forms at both 3.5 and 8.5% development (Mateus et al. 2014).

These hormone manipulations showed that early (3%), but not later (16%), injections led to a variety of phenotypes. In particular, they affected the area of some of the color rings, of some of the eyespots, on some of the wing surfaces. When expression of EcR was examined across these different wing pattern traits, there was no clear correlation between the traits affected and the presence/absence of EcR expression in that trait (Mateus et al. 2014). It is possible that, as in the injection experiments performed during the wandering stage of development, these injections are stimulating a second hormonal system, which in turn is exerting its effects on the eyespot phenotypes via its own receptor. Alternatively, given that only those eyespots and eyespot traits that were shown to be especially plastic responded to the hormone injections, it is possible that 20E is regulating directly the expression of these traits, but the developmental stage examined only captures effects on individuals with extended periods of sensitivity or heightened sensitivity to the hormone. Alternatively, lower basal levels of EcR observed across the whole

pupal wing epidermis are all that is required for 20E signaling to function at the period of development examined. None of the dorsal eyespots, however, responded to the injections (Mateus et al. 2014). This is likely because dorsal eyespot size plasticity, just as ventral eyespot size plasticity (Monteiro et al. 2015), is primarily controlled during the wandering stages of development (Bhardwaj et al. 2017), but perhaps these dorsal eyespots have fewer hormonal systems controlling their development, and cross talk between hormonal systems may have been minimized.

Going forward, future work on the physiological and genetic basis of wing pattern plasticity in any butterfly species should pay attention to a successive series of experiments that progressively narrows down the causative elements of trait plasticity. First, the critical period in development that is responsible for inducing trait plasticity should be identified using shifting experiments (see Monteiro et al. 2015). It is important to study each trait independently and not assume that the window of development controlling the features of a specific trait (say black ring of M1 eyespot on ventral forewing surface) will be the same as that controlling a similar but not identical trait (e.g., white center of the Cu1 eyespot on a different wing surface). Second, the physiological differences present at that stage (not later and not earlier) should be examined to pin down the physiological correlates that may underlie differences in trait development. Third, hormone depletion experiments (first) and hormone addition experiments (second) should be performed in order to mimic the physiological state of the two plastic forms, in a way that is independent of the environmental cue, to test causation. Here it is especially important to not raise hormone levels above those actually observed in the natural forms in order to avoid stimulating other hormone signaling systems in abnormal ways.

5.3 Evolution of Plasticity

Experiments on the evolution of plasticity in *B. anynana* have been of two types: microevolutionary population-level studies and macroevolutionary species-wide comparative studies. I will review these two types of experiments in turn.

The first type of study focused on testing whether genetic variation controlling the slope of a reaction norm, i.e., the sensitivity of ventral eyespot sizes to rearing temperature, was present in individuals of a single population. The initial rearing of different members of a family (representing similar genotypes) across different temperatures identified significant genetic variation for plasticity in a lab population of *B. anynana* (Windig 1994). In particular, variation in how each family responded to the same range of environments (temperatures) was captured via the presence of reaction norms with distinct slopes. However, further investigation concluded that this variation translated to minor changes to slopes when artificial selection was directly applied to the slope. These artificial selection experiments were of two types. The first type of experiment selected for steeper slopes by applying truncation selection for large eyespots at high temperature followed by

truncation selection on small eyespots at low temperature, in the following generation (trying to increase the slope) (Wijngaarden and Brakefield 2001). Alternatively, truncation selection was applied for small eyespots at high temperature and large eyespots at low temperature in the following generation (trying to decrease the slope) (Wijngaarden and Brakefield 2001). The second type of experiment split many individual families into four different rearing temperatures, examined what the reaction norms for each family across the three highest temperatures looked like, and then selected those families that had either the steeper or the shallower slopes by breeding from their siblings that were developing at the slowest (and lowest) temperature (Wijngaarden et al. 2002). Both types of experiment indicated that there was little to no genetic variation for slope of the reaction norms.

A different type of experiment, where artificial selection was applied to the size of the eyespots at a constant temperature (28 °C), followed by a subsequent examination of how these populations diverged in eyespot size across a range of rearing temperatures showed, again, no effects on slope of the reaction norms. All eyespots, regardless of starting size, became smaller with decreasing rearing temperature (Holloway and Brakefield 1995).

Despite the microevolutionary experiments above indicating little to no available genetic variation for selection on plasticity in a single lab population of *B. anynana*, the reality is that plasticity did evolve in this species, and this called for a broader exploration regarding the presence of plasticity in different populations of *B. anynana* and different species of *Bicyclus*.

5.4   Plasticity Across Populations and Species

Field collections have concluded that different environmental cues must be used to regulate eyespot size plasticity in different species of *Bicyclus* across Africa. When eyespot measurements of field-collected specimens were correlated with records of environmental variables, it was clear that species from southern regions, where temperature and humidity are positively correlated (warm wet season, cool dry season), use temperature as a cue to regulate eyespot size plasticity, but species from northern regions, where temperature and humidity are negatively correlated (warm dry season, cool wet season), are likely using humidity as the environmental cue that regulates eyespot size plasticity (Roskam and Brakefield 1999).

These predictions were confirmed when five species of *Bicyclus* from Southern Africa (from savannah and savannah-rainforest ecotones) and two from Equatorial Africa (rainforest) were reared in the lab under a common range of temperatures. All the species responded to temperature in a broadly similar way – ventral "exposed" eyespots became larger with increasing rearing temperature (Roskam and Brakefield 1996; Oostra et al. 2014). However, the savannah-rainforest species had steeper reaction norms relative to savannah or seasonal rainforest species (Roskam and Brakefield 1996).

Similar results were obtained in lab experiments where two southern populations of *B. anynana* (although from geographically distant locations in Malawi and South Africa) both developed larger ventral eyespots when reared at warmer temperatures, despite having diverged in absolute eyespot size at each of the temperatures (de Jong et al. 2010).

While common garden rearing experiments have yet to be performed with northern African population/species of *Bicyclus* butterflies, the general consensus emerging is that phenotypic plasticity for eyespot size, where exposed eyespots increase in size with increasing temperature, is an ancestral property for the genus *Bicyclus*, as well as for other related sayrine genera (Roskam and Brakefield 1996; Brakefield and Frankino 2009). When species move to equatorial regions where there is almost no fluctuation in temperature across the year, they do not lose their plastic response, presumably because there are few costs associated with maintaining the genetic mechanisms of temperature sensitivity in wing patterns (Oostra et al. 2014).

Broader explorations of eyespot plasticity are now necessary, beyond the satyrids, for a more complete understanding of the evolution of eyespot size plasticity. Preliminary data (S. Bhardwaj, unpublished) indicates that many nymphalid butterflies outside the satyrids show the exact opposite pattern of plasticity in eyespot size in relation to rearing temperature. High rearing temperatures lead to smaller eyespots, instead of larger eyespots. The ecological significance of these patterns as well as their underlying physiological mechanisms needs to be examined in detail in the future for a more comprehensive examination of how plasticity in eyespots evolved.

5.5 Conclusions

The ecological significance of wing pattern plasticity in *Bicyclus anynana* is becoming increasingly well understood. In particular, exposed eyespots serve a cryptic function in the dry season, whereas they serve a deflection function in the wet season. Nonexposed eyespots serve a sexual signaling function and display their own patterns of plasticity, distinct from those of exposed eyespots. In addition, patterns of plasticity for each eyespot and for each of the color components within an eyespot are very eyespot-specific and need to be studied in isolation. The physiological basis of eyespot size plasticity in this species, unfortunately, focused for a very long time on a developmental period of low temperature sensitivity (the early pupal stage) instead of the more highly sensitive wandering larval stage of development. So, much of the early work in this system needs to be read and interpreted with caution. More recent experiments have clarified the developmental window and the physiological basis for size plasticity of both dorsal and ventral eyespots, and we have only begun to explore how different homologous wing pattern elements respond to the same environmental cue in different ways. Still, much work still remains to be done. For instance, as pointed out above, different

species living in different environments are likely to use different cues to regulate homologous wing pattern elements. However, we still don't know which cues are used (besides temperature) and how they affect wing pattern development. We still don't understand how temperature regulates hormone titers in *B. anynana* and how 20E signaling regulates eyespot size, and we have no idea of the role of epigenetic processes, if any, on the regulation of this process. Finally, comparative work across species is necessary to understand when 20E hormone titers became regulated by rearing temperature at the wandering stage of development, when the ecdysone receptor became recruited to eyespot centers, making them sensitive to fluctuating 20E titers, and when genes from the eyespot gene regulatory network became sensitive to 20E signaling.

References

Bear A, Monteiro A (2013) Male courtship rate plasticity in the butterfly Bicyclus anynana is controlled by temperature experienced during the pupal and adult stages. PLoS One 8(5): e64061. doi:10.1371/journal.pone.0064061. PubMed PMID: WOS:000320362700095

Beldade P, Mateus ARA, Keller RA (2011) Evolution and molecular mechanisms of adaptive developmental plasticity. Mol Ecol 20(7):1347–1363. doi:10.1111/j.1365-294X.2011.05016.x. PubMed PMID: WOS:000288705300006

Bhardwaj S, Prudic KL, Bear A, Das Gupta M, Cheong WF, Wenk MR, et al. (2017) Sex differences in 20-hydroxyecdysone hormone levels control sexual dimorphism in butterfly wing patterns. bioRxiv, 124834.

Bradshaw AD (1956) Evolutionary significance of phenotypic plasticity in plants. In: Caspari EW (ed) Adv Genet. 13. Academic, New York, pp 115–155

Brakefield PM, Frankino WA (2009) Polyphenisms in Lepidoptera: multidisciplinary approaches to studies of evolution and development. In: Whitman DW, Ananthakrishnan TN (eds) Phenotypic plasticity in insects mechanisms and consequences. Science Publishers, Plymouth, pp 281–312

Brakefield PM, Larsen TB (1984) The evolutionary significance of dry and wet season forms in some tropical butterflies. Biol J Linn Soc 22:1–12

Brakefield PM, Mazzotta V (1995) Matching field and laboratory environments – effects of neglecting daily temperature-variation on insect reaction norms. J Evol Biol 8(5):559–573. doi:10.1046/j.1420-9101.1995.8050559.x. PubMed PMID: WOS:A1995RY61500002

Brakefield PM, Reitsma N (1991) Phenotypic plasticity, seasonal climate and the population biology of Bicyclus butterflies (Satyridae) in Malawi. Ecol Entomol 16:291–303. doi:10.1111/j.1365-2311.1991.tb00220.x

Brakefield P, Gates J, Keys D, Kesbeke F, Wijngaarden P, Monteiro A et al (1996) Development, plasticity and evolution of butterfly eyespot patterns. Nature 384(6606):236–242. doi:10.1038/384236a0. PubMed PMID: WOS:A1996VU38100042

Brakefield PM, Kesbeke F, Koch PB (1998) The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. Am Nat 152:853–860

Brunetti C, Selegue J, Monteiro A, French V, Brakefield P, Carroll S (2001a) The generation and diversification of butterfly eyespot color patterns. Curr Biol 11(20):1578–1585. doi:10.1016/S0960-9822(01)00502-4. PubMed PMID: WOS:000171651700015

Brunetti CR, Selegue JE, Monteiro A, French V, Brakefield PM, Carroll SB (2001b) The generation and diversification of butterfly eyespot color patterns. Curr Biol 11:1578–1585

Condamin M (1973) Monographie du genre *Bicyclus* (Lepidoptera, Satyridae). IFAN, Dakar

Costanzo K, Monteiro A (2007) The use of chemical and visual cues in female choice in the butterfly Bicyclus anynana. Proc R Soc Lond B Bio 274(1611):845–851. doi:10.1098/rspb.2006.3729. PubMed PMID: WOS:000243842100012

de Jong G (2005) Evolution of phenotypic plasticity: patterns of plasticity and the emergence of ecotypes. New Phytol 166(1):101–117. doi:10.1111/j.1469-8137.2005.01322.x. PubMed PMID: WOS:000227390500011

de Jong MA, Kesbeke F, Brakefield PM, Zwaan BJ (2010) Geographic variation in thermal plasticity of life history and wing pattern in Bicyclus anynana. Clim Res 43(1–2):91–102. doi:10.3354/cr00881. PubMed PMID: WOS:000280830000010

Dinan L, Whiting P, Girault JP, Lafont R, Dhadialla TS, Cress DE et al (1997) Cucurbitacins are insect steroid hormone antagonists acting at the ecdysteroid receptor. Biochem J 327:643–650. PubMed PMID: ISI:A1997YF86500003

Dion E, Monteiro A, Yew JY (2016) Phenotypic plasticity in sex pheromone production in *Bicyclus anynana* butterflies. Sci Report 6:39002. doi:10.1038/srep39002

Everett A, Tong XL, Briscoe AD, Monteiro A (2012) Phenotypic plasticity in opsin expression in a butterfly compound eye complements sex role reversal. BMC Evol Biol 12:232. doi:10.1186/1471-2148-12-232. PubMed PMID: WOS:000313877800001

Fischer K, Brakefield PM, Zwaan BJ (2003a) Plasticity in butterfly egg size: why larger offspring at lower temperatures? Ecology 84(12):3138–3147. doi:10.1890/02-0733

Fischer K, Eenhoorn E, Bot AN, Brakefield PM, Zwaan BJ (2003b) Cooler butterflies lay larger eggs: developmental plasticity versus acclimation. Proc R Soc B 270(1528):2051–2056. PubMed PMID: 14561294

French V, Brakefield PM (1992) The development of eyespot patterns on butterfly wings: morphogen sources or sinks? Development 116:103–109

Ho S, Schachat SR, Piel WH, Monteiro A (2016) Attack risk for butterflies changes with eyespot number and size. R Soc Open Sci 3:150614

Holloway GJ, Brakefield PM (1995) Artificial selection of reaction norms of wing pattern elements in Bicyclus-anynana. Heredity 74:91–99. PubMed PMID: ISI:A1995QA25300011

Koch PB, Buckmann D (1987) Hormonal-control of seasonal morphs by the timing of ecdysteroid release in Araschnia levana L (Nymphalidae, Lepidoptera). J Insect Physiol 33(11):823–829. PubMed PMID: ISI:A1987K176700006

Koch PB, Brakefield PM, Kesbeke F (1996) Ecdysteroids control eyespot size and wing color pattern in the polyphenic butterfly *Bicyclus anynana* (Lepidoptera, Satyridae). J Insect Physiol 42:223–230

Kooi RE (1995) The effect of food plant quality on wing pattern induction in the tropical butterfly Bicyclus anynana (Satyrinae). In: Sommeijer MJ, Francke PJ (eds), pp 107–112

Kooi RE, Brakefield PM. The critical period for wing pattern induction in the polyphenic tropical butterfly Bicyclus anynana (Satyrinae). J Insect Physiol 1999;45(3):201–212. PubMed

Lyytinen A, Brakefield PM, Mappes J (2003) Significance of butterfly eyespots as an anti-predator device in ground-based and aerial attacks. Oikos 100(2):373–379. PubMed PMID: ISI:000181854900018

Lyytinen A, Brakefield PM, Lindstrom L, Mappes J (2004) Does predation maintain eyespot plasticity in *Bicyclus anynana*? Proc R Soc Lond B Bio 271(1536):279–283. PubMed PMID: ISI:000188694400010

Macias-Munoz A, Smith G, Monteiro A, Briscoe AD (2016) Transcriptome-wide differential gene expression in Bicyclus anynana butterflies: female vision-related genes are more plastic. Mol

Biol Evol 33(1):79–92. doi:10.1093/molbev/msv197. PubMed PMID: WOS:000369992600006

Mateus ARA, Marques-Pita M, Oostra V, Lafuente E, Brakefield PM, Zwaan BJ et al (2014) Adaptive developmental plasticity: compartmentalized responses to environmental cues and to corresponding internal signals provide phenotypic flexibility. BMC Biol 12:97. doi:10.1186/s12915-014-0097-x. PubMed PMID: WOS:000348150100001

Monteiro A, Brakefield P (1994) French v. The evolutionary genetics and developmental basis of wing pattern variation in the butterfly bicyclus-anynana. Evol Int J Org Evol 48(4):1147–1157. doi:10.2307/2410374. PubMed PMID: WOS:A1994QE32300018

Monteiro A, Glaser G, Stockslager S, Glansdorp N, Ramos D (2006) Comparative insights into questions of lepidopteran wing pattern homology. BMC Dev Biol 6. doi:10.1186/1471-213X-6-52. PubMed PMID: WOS:000242189200001

Monteiro A, Tong XL, Bear A, Liew SF, Bhardwaj S, Wasik BR et al (2015) Differential expression of ecdysone receptor leads to variation in phenotypic plasticity across serial homologs. PLoS Genet 11(9). doi:10.1371/journal.pgen.1005529. PubMed PMID: WOS:000362269000043

Moran NA (1992) The evolutionary maintenance of alternative phenotypes. Am Nat 139(5):971–989. PubMed PMID: ISI:A1992HU07100005

Nijhout HF (1980) Ontogeny of the color pattern on the wings of *Precis coenia* (Lepidoptera: Nymphalidae). Dev Biol 80:275–288

Nijhout HF (1999) Control mechanisms of polyphenic development in insects. Bioscience 49(3):181–192. doi:10.2307/1313508

Nijhout HF (2003) Development and evolution of adaptive polyphenisms. Evol Dev 5(1):9–18. doi:10.1046/j.1525-142X.2003.03003.x. PubMed PMID: WOS:000179925400003

Oliver JC, Ramos D, Prudic KL, Monteiro A (2013) Temporal gene expression variation associated with eyespot size plasticity in Bicyclus anynana. PLoS One 8(6). doi:10.1371/journal.pone.0065830. PubMed PMID: WOS:000320440500052

Olofsson M, Jakobsson S, Wiklund C (2013) Bird attacks on a butterfly with marginal eyespots and the role of prey concealment against the background. Biol J Linn Soc 109(2):290–297. doi:10.1111/bij.12063. PubMed PMID: WOS:000318809500004

Oostra V, de Jong MA, Invergo BM, Kesbeke F, Wende F, Brakefield PM et al (2011) Translating environmental gradients into discontinuous reaction norms via hormone signalling in a polyphenic butterfly. Proc R Soc B Biol Sci 278(1706):789–797. doi:10.1098/rspb.2010.1560. PubMed PMID: ISI:000286507400021

Oostra V, Brakefield PM, Hiltemann Y, Zwaan BJ, Brattstrom O (2014) On the fate of seasonally plastic traits in a rainforest butterfly under relaxed selection. Ecol Evol 4(13):2654–2667. doi:10.1002/ece3.1114. PubMed PMID: WOS:000339494900004

Orme MH, Leevers SJ Flies on steroids: the interplay between ecdysone and insulin signaling. Cell Metab 2(5):277–278. doi:10.1016/j.cmet.2005.10.005

Prudic KL, Jeon C, Cao H, Monteiro A (2011) Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. Science 331(6013):73–75. doi:10.1126/science.1197114. PubMed PMID: WOS:000285974000038

Prudic KL, Stoehr AM, Wasik BW, Monteiro A (2015) Invertebrate predators attack eyespots and promote the evolution of phenotypic plasticity. Proc R Soc Lond B Bio 282(1798):20141531

Robertson K, Monteiro A (2005) Female Bicyclus anynana butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. Proc R Soc Lond B Bio 272(1572):1541–1546. doi:10.1098/rspb.2005.3142. PubMed PMID: WOS:000231504300003

Roskam JC, Brakefield PM (1996) A comparison of temperature-induced polyphenism in African *Bicyclus* butterflies from a savannah-rainforest ecotone. Evol Int J Org Evol 50:2360–2372

Roskam JC, Brakefield PM (1999) Seasonal polyphenism in Bicyclus (Lepidoptera: Satyridae) butterflies: different climates need different cues. Biol J Linn Soc 66(3):345–356. doi:10.1111/j.1095-8312.1999.tb01895.x. PubMed PMID: WOS:000079376200005

Rountree DB, Nijhout HF (1995) Hormonal control of a seasonal polyphenism in precis coenia (Lepidoptera: Nymphalidae). J Insect Physiol 41(11):987–992

Stearns SC (1989) The evolutionary significance of phenotypic plasticity. Bioscience 39 (7):436–445. PubMed PMID: ISI:A1989AC84800005

Vlieger L, Brakefield PM (2007) The deflection hypothesis: eyespots on the margins of butterfly wings do not influence predation by lizards. Biol J Linn Soc 92(4):661–667. PubMed PMID: WOS:000251414500006

West-Eberhard MJ (2003) Developmental plasticity and evolution. Oxford University Press, New York, 794 p

Westerman E, Monteiro A (2016) Rearing temperature influences adult response to changes in mating status. PLoS One 11(2):e0146546. doi:10.1371/journal.pone.0146546. PubMed PMID: WOS:000370046600012

Wijngaarden PJ, Brakefield PM (2001) Lack of response to artificial selection on the slope of reaction norms for seasonal polyphenism in the butterfly Bicyclus anynana. Heredity 87:410–420. doi:10.1046/j.1365-2540.2001.00933.x. PubMed PMID: WOS:000172693900004

Wijngaarden PJ, Koch PB, Brakefield PM (2002) Artificial selection on the shape of reaction norms for eyespot size in the butterfly Bicyclus anynana: direct and correlated responses. J Evol Biol 15(2):290–300. doi:10.1046/j.1420-9101.2002.00380.x. PubMed PMID: WOS:000174709000012

Windig JJ (1994) Reaction norms and the genetic-basis of phenotypic plasticity in the wing pattern of the butterfly Bicyclus anynana. J Evol Biol 7(6):665–695. doi:10.1046/j.1420-9101.1994.7060665.x. PubMed PMID: ISI:A1994PV78800002

Windig JJ, Brakefield PM, Reitsma N, Wilson JGM (1994) Seasonal polyphenism in the wild: survey of wing patterns in five species of *Bicyclus* butterflies in Malawi. Ecol Entomol 19:285–298

Zera AJ (2007) Endocrine analysis in evolutionary-developmental studies of insect polymorphism: hormone manipulation versus direct measurement of hormonal regulators. Evol Dev 9 (5):499–513. PubMed PMID: ISI:000249321200009